

## Early Detection of Anti-HCc Antibody in Acute Hepatitis C Virus (HCV) by Western Blot (Immunoblot) Using a Recombinant HCV Core Protein Fragment

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Crude extract from *Escherichia coli* which expressed a recombinant protein containing amino acids 2 to 127 of the hepatitis C virus (HCV) core protein was used to detect the antibody against HCV core protein (anti-HCc). After electrophoretic separation of proteins from the extract, Western blot (immunoblot) analysis was performed with the serum samples. This method was compared with a commercially available second-generation enzyme immunoassay (EIA) which employed synthetic peptides corresponding to highly antigenic segments of both structural and nonstructural portions of HCV. Also, reverse transcription PCR for HCV RNA was used for comparison. Seventy-two serum samples from three groups of patients were tested. Groups I and II represented healthy subjects and subjects with acute hepatitis A or B, respectively. Group III included patients with newly acquired acute hepatitis C. By Western blot analysis, 31 of 31 (100%) samples from group I were negative for anti-HCc antibody, whereas 4 of 22 (18%) samples from group II were positive for anti-HCc. One of these four samples was also positive for anti-HCV antibody by the second-generation EIA (1 of 22 [4.5%]). Among 19 patients diagnosed with newly acquired acute hepatitis C, 4 (21%) were positive for anti-HCV by the second-generation EIA, whereas 12 of 19 (63%) were positive for anti-HCc by Western blot analysis. Of EIA-positive subjects, 4 of 4 (100%) were also positive for anti-HCc by Western blot analysis, whereas among EIA-negative subjects, 8 of 15 (53%) were positive. For HCV RNA detected by reverse transcription PCR, 15 of 19 (80%) of this group of samples were positive. Strikingly, the peak bilirubin level for patients with EIA-negative and Western blot-positive results is significantly higher than that for patients with consistent EIA and Western blot results (22.7 versus 7.2 mg/dl). A series of serum samples from a patient with concurrent hepatitis B and C viral infection was also studied by both tests. Although anti-HCc persisted throughout the course of infection, anti-HCV by EIA converted from negative to positive 20 days after admission and then converted back to negative 30 days later.

Hepatitis C virus (HCV) is a single-stranded, linear RNA virus which causes hepatitis, especially through blood transfusion (11). Isolation of the HCV genome enables a researcher to develop diagnostic assays for hepatitis C (8). These techniques have greatly improved our ability to control this disease. Several different tests have been developed and are commercially available. First-generation tests were developed by using a segment of protein from the nonstructural portion of the HCV polyprotein to detect the antibody against it. For example, the peptide C100-3 originates from the NS4 region of the HCV polyprotein and is used for antibody detection in the early test developed by Chiron Corporation (16). After the whole HCV genome had been cloned, the importance of HCV structural proteins was noted (3, 9, 31). The second-generation tests developed later make use of more than one fragment of the HCV polyprotein, including both structural and nonstructural parts as target antigens for the tests, and thus achieve higher sensitivity (3, 15, 31, 33). The methods employed by second-generation tests vary from one to another, for example, enzyme-linked assays (enzyme immunoassay [EIA] and enzyme-linked immunosorbent assay) (3, 31, 33) and recombinant immunoblot assays (31). The sources of protein fragments (antigens) were also different. For example, either recombi-

nant fusion protein or synthetic peptides can be used, and each method claims to have its advantages (2, 6, 7, 14). The sensitivities and specificities among different tests are not the same. From recent reports, the second-generation, four-strip recombinant immunoblot assay, which allows us to visualize the results of four different reactions, seems to be the most reliable test (21, 35). Both sensitivity and specificity are high when reverse transcription PCR (RT-PCR) is used as a standard. Unfortunately, this kit is not widely used clinically because of economic considerations. Instead, the second-generation EIA is more widely accepted for clinical use, though its specificity is questionable compared with that of the second-generation recombinant immunoblot assay (22).

Early diagnosis of acute hepatitis C has been a problem for clinicians. The appearance of anti-HCV antibody by EIA usually occurs quite late after onset of the disease. In addition, because intermittent and transient viremias have been reported, RT-PCR for HCV RNA is also not a very reliable test in the acute phase of this disease (1, 10, 12). A better test is required for this group of patients to provide earlier diagnosis. On the basis of the idea that immunoblotting could be more sensitive and reliable than other enzyme-based assays, we developed an immunoblot assay to detect the HCV core (HCc) protein. We first expressed a recombinant fragment of HCc protein in *Escherichia coli*. This protein can be separated from other impurities by polyacrylamide gel electrophoresis. Subsequently, serum can be analyzed for the anti-HCc antibody by

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TABLE 1. Clinical and laboratory data of patients studied

Group	No. of subjects	No. of subjects by sex (male/female)	Age <sup>a</sup> (yr)	Peak level <sup>c</sup>	
				ALT (IU/liter)	Bilirubin (mg/dl)
I	31	20/11	41 ± 17	15 ± 5	ND <sup>b</sup>
II	22	10/12	29 ± 10	1,570 ± 952	5.7 ± 4.2
III	19	10/9	42 ± 16	1,347 ± 661	13.7 ± 11.5

<sup>a</sup> Data are means ± one standard deviation.

<sup>b</sup> ND, not done.

Western blot (immunoblot). This method was compared with a second-generation EIA which used synthetic peptides from structural and nonstructural parts of the HCV polyprotein and claimed to have both high sensitivity and specificity (2, 14). The subjects included in our study had newly acquired acute hepatitis C, while patients with acute hepatitis A or B were used as a control.

In Taiwan, a high carrier rate of HBV has been noted (29). Thus, double infection by HBV and HCV is not uncommon (18). This creates a special situation in our medical practice. In this report, we also compare both methods in the case of an acute hepatitis C infection with an HBV carrier background. Results indicate that our Western blot method can be complementary to the second-generation EIA in detecting anti-HCV antibodies in serum samples from acute HCV-infected patients. The significance and alteration of our clinical judgment of disease status by this method are discussed.

#### MATERIALS AND METHODS

**Subjects.** Serum samples were collected by a different team than the one performing Western blotting. Three groups of patients were included. Group I represented a healthy control group (31 subjects), all with normal alanine transaminase (ALT) levels and no known history or symptoms of hepatitis. Group II included patients with acute hepatitis A or B (22 subjects). Acute hepatitis A (eight subjects) was diagnosed from an ALT level >400 IU/liter, positive immunoglobulin M (IgM) for anti-HAV, negative IgM for anti-HBc, and negative HBV surface antigen (HBsAg). Acute hepatitis B (14 subjects) was diagnosed from an ALT level >400 IU/liter, positive IgM for anti-HBc, and negative IgM for anti-HAV. HBsAg converted from positive to negative in 12 cases. For the other two cases, HBsAg was always negative from the first visit. Group III represented patients with acute hepatitis C (19 subjects). Diagnosis of acute hepatitis C was determined by (i) seroconversion of anti-HCV from negative to positive in serial follow-ups by second-generation EIA (15 patients) or (ii) results negative for anti-HCV by second-generation EIA but positive for HCV RNA by RT-PCR on at least one occasion if the follow-up period was not long enough for anti-HCV seroconversion (4 patients). Also, IgM anti-HAV, IgM anti-HBc, and HBsAg were all negative in all of these cases. The serum samples subjected to this study were the first available samples taken upon presentation of the patients.

The basic data of these patients are shown in Table 1. The mean peak ALT level for group I (15 IU/liter) was in the normal range, while those for the other two groups were over 1,000 IU/liter (1,570 and 1,347 IU/liter). The mean peak bilirubin level for group III (13.7 mg/dl) was higher than that for group II (5.7 mg/dl), but the difference was not statistically significant. The higher mean level and wider range were caused by four samples with high bilirubin levels. The average age (29

years old) of group II was younger than those of the others (41 and 42 years old). Six of the patients in group III had received blood transfusions prior to acute hepatitis (see Table 3, patients A, E, F, P, Q, and R).

**RT-PCR.** For detection of HCV RNA in patient serum samples, two sets of primers were designed according to the published Taiwan strain for nested PCR (4). The noncoding region was selected as the PCR target (17, 20, 34). The outer primers were 5' ACTCCACCATAGATCACTCC 3' and 5' CATGGTGCACGGTCTACGAG 3' (nucleotide -318 to -299 [sense] and 3 to -16 [antisense]). The inner primers were 5' GGAAGTACTGTCTTCACGCA 3' and 5' AACACTACTCGGCTAGCAGT 3' (nucleotide -291 to -272 [sense] and -77 to -96 [antisense]). Serum (100 µl) was mixed with 600 µl of extraction buffer (4 M guanidium thiocyanate, 2% sarcosyl, 0.5 M mercaptoethanol, 5 µg of tRNA, 5 mM sodium acetate [pH 6]). After vortex, phenol-chloroform extraction was done once and then nucleic acid was precipitated with an equal volume of isopropyl alcohol. The pellet was vacuum dried. For RT, 10 U of RNasin (HT Biotechnology, Ltd., Cambridge, England) and 50 ng of random primer (hexamers) (provided by TIB Molbiol, Berlin, Germany) were added together with 10.5 µl of pure water to the pellet. The mixture was warmed to 70°C for 5 min. Then 1 µl of 5 mM deoxynucleoside triphosphate (dNTP), 10 U of RNasin, 20 µg of bovine serum albumin (BSA), and 1 µl of Moloney murine leukemia virus reverse transcriptase (20 U/µl) (Gibco BRL, Life Technologies, Inc., Gaithersburg, Md.) together with the provided reaction buffer were added. The mixture was brought to room temperature for 15 min, and then the reaction was carried out at 37°C for 90 min. After reverse transcription, PCR was done in two steps with two sets of primers. For primary PCR, the reaction was carried out in 200 µM dNTP-50 pmol of each primer (outer primers)-2 U of Super Tag (HT Biotechnology, Ltd.) together with the provided PCR reaction buffer. The reaction was performed with a DNA thermal cycler (Perkin-Elmer Cetus, Emeryville, Calif.) with a step cycle program at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s for 32 cycles. For secondary PCR, 5 µl from the first PCR product was used as the source of the DNA template. The inner primers instead of the outer primers were used for the reaction. The final product was analyzed on a 1.8% agarose gel.

**Construction of the HCc protein expression plasmid pS68-C.** A fragment of the HCc protein gene was isolated from patient serum by RT-PCR as described above except only the primary PCR reaction was done and the primers used were 5' CCGTGCGAATTCAGCACGAAT 3' and 5' GTCGGC GAATTCTCACGTAAG 3' (modified from nucleotide -8 to 12 [sense] and 396 to 376 [antisense]). The PCR product was digested with *EcoRI* and then ligated to an *E. coli* expression vector, pET-3a'. pET-3a' was derived from pET-3a with the following modification (25). The original *EcoRI* site was

mutated, and a sequence derived from the pFLAG vector (FLAG Biosystem; International Biotechnologies, Inc.) was inserted into the *Nde*I site (28). This sequence included the new *Eco*RI site into which the PCR product was inserted. The final construct, pS68-C, resulted in a gene encoding a fusion protein with 17 leading amino acids, MDYKDDDDKARRAS VEF, linked in frame with amino acids 2 to 127 of HC<sub>c</sub> protein. A stop codon in the 3' PCR primer (antisense) right after the codon of amino acid 127 was engineered.

**Expression of the HC<sub>c</sub> protein fragment in *E. coli*.** pS68-C was used to transform *E. coli* BL21(DE3)pLysS, which contained a lysogenized lambda phage that carried the gene for T7 RNA polymerase under the control of the *lac* UV5 promoter and the plasmid pLysS that expressed low levels of T7 lysozyme, a natural inhibitor of T7 RNA polymerase (28). *E. coli* was cultured in Luria-Bertani medium containing 50 µg of ampicillin per ml and 20 µg of chloramphenicol per ml for 15 h until the optical density (OD) reached 0.6, and then 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added. After 2 h, bacteria were harvested and resuspended in 6 ml of solution containing 25% sucrose, 50 mM Tris (pH 8), and 5 mg of lysozyme (27). The mixture was placed on ice for 5 min, and then 2.5 ml of 0.25 M EDTA was added. Subsequently, 10 ml of lysis buffer (1% Triton X-100, 0.4% sodium deoxycholate, 50 mM Tris, 62.5 mM EDTA) was added and mixed for 10 min on ice. One milliliter of 1 M MgCl<sub>2</sub> and 2 mg of DNase I were added. The mixture was stored at 37°C for 1 h before it was centrifuged at 10,000 × *g* for 10 min. Crude extract (supernatant) was mixed with an equal volume of 2× sample buffer (0.135 M Tris-HCl [pH 6.8], 6% sodium dodecyl sulfate, 20% glycerol, 10% mercaptoethanol), heated to 65°C for 5 min, and then stored at -20°C until used.

**Western blot and serum analysis.** Crude extract obtained as described above was subjected to polyacrylamide gel electrophoresis and then electrotransferred to a nitrocellulose membrane. The nitrocellulose membrane was incubated with 1% BSA in Tris-buffered saline (TBS) (10 mM Tris-HCl [pH 7], 0.15 M NaCl) for 3 h at room temperature and then with serum from a patient at a 1:1,500 dilution with the same buffer as the diluent (1% BSA in TBS). After 3 h, the paper was washed twice with 0.1% Nonidet P-40 in TBS. Then it was incubated with the secondary antibody (alkaline phosphatase-conjugated goat anti-human antibody) (Jackson Immunology Research Lab., Inc., West Grove, Pa.) for 1 h. The membrane was subsequently washed twice more with TBS containing 0.1% Nonidet P-40, and the signal was developed with Western Blue, a stabilized substrate for alkaline phosphatase (Promega Corporation, Madison, Wis.).

The second-generation EIA used in this study was performed according to the protocol provided by the manufacturer (United Biomedical, Inc., Lake Success, N.Y.). HBsAg, HBV e antigen (HBeAg), anti-HAV IgM, and anti-HBV IgM were detected by commercially available radioimmunoassays (AUSRIA-II, HBe (rDNA), and HAVAB-M [Abbott Laboratories, North Chicago, Ill.] and SB-CORE-IgM [Sorin Biomedica, Torino, Italy], respectively).

## RESULTS

**Isolation and expression of recombinant HC<sub>c</sub> protein fragment.** The core gene fragment was isolated from a serum sample tested to be positive for HCV RNA by RT-PCR. The fragment isolated with the primers described in Materials and Methods was 405 bp, which included the coding region of HC<sub>c</sub> protein from amino acid 1 to 127. The nucleotide sequence had 98% homology with the prototype sequence (type I) isolated

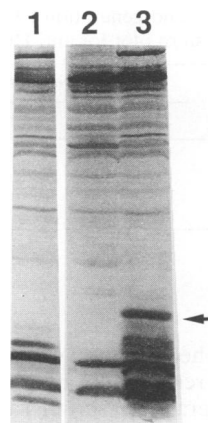


FIG. 1. Western blot analysis with crude extract from *E. coli* and serum samples from a healthy subject and a patient with HCV infection. Lanes: 1, crude extract from BL21(DE3)pLysS transformed by pS68-C with a serum sample from a healthy subject; 2, crude extract from BL21(DE3)pLysS transformed by a control plasmid (pET-3a') with a serum sample from a patient with chronic HCV infection; 3, the same crude extract used in lane 1 with the same serum used in lane 2. The arrow marks the position of the band for the recombinant HC<sub>c</sub> protein fragment (15 kDa).

by Chiron Corporation (8, 24) and 89% homology with the Taiwanese HCV sequence (type II) (4, 24). However, the amino acid sequence homology was 97 and 92% with the prototype and Taiwanese genomes, respectively. This gene fragment was then cut with *Eco*RI, which was engineered on both ends, and inserted into an expressing vector. The resulting plasmid, pS68-C, expressed a protein containing 17 amino acid residues from the pFLAG vector and amino acid 2 to 127 of the HC<sub>c</sub> protein sequence. The plasmid was transformed into *E. coli* BL21(DE3)pLysS for protein expression. After IPTG induction, the crude cell lysate was collected and subjected to polyacrylamide gel electrophoresis prior to Western blotting. In Fig. 1, lane 2 contained crude extract from *E. coli* transformed by a control plasmid and blotted with serum from a chronically HCV-infected patient. The serum had been tested to be positive for HCV RNA by RT-PCR. The crude extract from *E. coli* transformed by plasmid pS68-C, which expressed recombinant HC<sub>c</sub> protein, was incubated with the same positive serum for Western blotting. The results are shown in lane 3 of Fig. 1. The same extract used in lane 3, which contained the recombinant HC<sub>c</sub> protein, was blotted with a serum sample from a healthy subject (Fig. 1, lane 1). A prominent band in the predicted position of about 15 kDa appeared only in lane 3 of Fig. 1. Several bands that appeared at the upper- and lower-most parts of all three lanes were due to nonspecific background reactions. The nature of the bands migrating faster than the specific band of 15 kDa in lane 3 (Fig. 1) was not clear. It could be due to degradation of the recombinant protein. These bands were not always reproducible. This result indicated that the 15-kDa band in lane 3 of Fig. 1 represented the specific band for recombinant HC<sub>c</sub> protein.

**Tests with serum samples from patients with acute hepatitis.** Three groups of serum samples were submitted to this study. Each Western blot analysis included positive and negative controls. The positive control serum was obtained from a patient with chronic hepatitis C, which was confirmed by repeatedly testing positive for anti-HCV antibody by EIA and positive for HCV RNA by RT-PCR during the follow-up. The negative control serum was obtained from a healthy subject

TABLE 2. Results of second-generation EIA for anti-HCV and of Western blot for anti-HCc

Group	No. of subjects	No. positive (%) by:		
		2 <sup>nd</sup> EIA	Immunoblot	RT-PCR
I	31	ND <sup>b</sup>	0 (0)	0 (0)
II	22	1 (4.5)	4 (18)	0 (0)
III	19	4 (21.0)	12 (63)	15 (80)

<sup>a</sup> 2<sup>nd</sup>, second generation.<sup>b</sup> ND, not done.

who had no history of hepatitis and was negative for anti-HCV and HCV RNA. The results were read without knowledge of the sources of the sera. For serum samples from group I (healthy controls), all 31 samples were negative for anti-HCc when analyzed by this method (Table 2). For group II (acute hepatitis A or B), 4 of 22 samples were positive for anti-HCc by Western blot. Also, one of these four was positive for anti-HCV by second-generation EIA (OD = 0.52 [cutoff = 0.16]). All four were negative for HCV RNA by RT-PCR. The sample that tested positive by both EIA and Western blot was originally diagnosed as acute hepatitis B infection by serological markers. The other three patients with a positive anti-HCc reaction were originally diagnosed as patients with acute hepatitis B infection in two cases and with acute hepatitis A infection in the other by serological markers. For samples from group III (acute hepatitis C), 12 of 19 (63%) were positive for anti-HCc by Western blot analysis, whereas 4 of 19 (21%) were positive for anti-HCV by second-generation EIA (Table 2). In all four of the EIA-positive cases, the test for anti-HCc was also positive. For the seven samples with negative anti-HCc results, the EIAs were also negative. From HCV RNA analysis of this group of patients, 15 of 19 (80%) were positive by RT-PCR (Table 3). In the four remaining cases, the test for HCV RNA was negative despite repeated attempts by RT-PCR with the same serum sample. Of these four cases, two were negative both for anti-HCc by Western blot and for

anti-HCV by EIA, though the EIA became positive during follow-up. Both of the other two cases were positive for anti-HCc, but only one was positive for anti-HCV by EIA. Therefore, there was one serum sample which was negative by both EIA and RT-PCR but positive for anti-HCc. This case was later seroconverted to positive for anti-HCV by EIA.

The intervals between peak ALT elevation and the time of blood sampling for Western blotting varied significantly among our patients. It ranged from 19 days before the peak ALT level to 90 days after the peak. For samples that were negative by EIA and positive by Western blot (patients F to L and S) (Table 3), sera were obtained within -19 to 18 days after the peak ALT level except for patient G (51 days). The four patients with negative RT-PCR results were checked -14 to 2 days after the peak ALT level. Strikingly, the mean peak bilirubin level for patients with EIA-negative and Western blot-positive results was higher ( $22.7 \pm 12.3$  mg/dl) than those for patients with EIA and Western blot double-positive results ( $5.6 \pm 3.6$  mg/dl), double-negative results ( $8.2 \pm 4.9$  mg/dl), or both double-positive and -negative results ( $7.2 \pm 4.5$  mg/dl) ( $P = 0.0005, 0.0002, \text{ and } 0.0008$ , respectively, by the Student *t* test).

**Serum samples from a patient with HBV and HCV double viral infection.** Serial serum samples from a chronic HBV carrier with acute HCV superinfection were assayed. This 28-year-old female suffered from jaundice, malaise, and tea-colored urine 1 month before visiting our hospital. She was admitted because of lethargy and change of personality. The results of serial serological tests are summarized in Fig. 2. Tests for IgM anti-HBc and IgM anti-HAV were both negative and the HBsAg test was positive when she was admitted. The second-generation EIA for anti-HCV antibody showed seroconversion from negative to positive 20 days after admission. Subsequently, HBeAg was converted to negative and the anti-HBe antibody became positive after the attack of acute hepatitis C. The biopsy done on the 20th day after admission showed acute hepatitis with bridging hepatic necrosis. In addition, bile ductular proliferation and bile plugging with

TABLE 3. Clinical and laboratory data for patients in group III

Patient	Sex <sup>a</sup>	Age (yr)	Peak level		Result by:			Time of blood sampling (days after peak ALT)
			ALT (IU/liter)	Bilirubin (mg/dl)	2 <sup>nd</sup> EIA	Western blot	RT-PCR	
A	F	63	553	1.2	-	-	+	2
B	M	62	2,054	17.0	-	-	+	22
C	F	58	1,177	9.8	-	-	+	26
D	M	41	1,076	5.8	-	-	+	90
E	F	25	851	5.5	-	-	+	-11
F	F	50	548	1.3	-	+	+	-19
G	M	51	1,049	21.4	-	+	+	51
H	M	67	1,528	45.1	-	+	+	2
I	M	51	771	19.8	-	+	+	1
J	M	32	1,872	17.5	-	+	+	3
K	M	50	943	19.9	-	+	+	1
L	F	37	2,426	27.8	-	+	+	1
M	M	29	1,529	6.5	+	+	+	9
N	F	26	1,531	6.8	+	+	+	0
O	F	33	1,263	8.6	+	+	+	60
P	M	74	2,580	10.2	-	-	-	2
Q	F	22	1,051	7.9	-	-	-	0
R	F	32	448	0.4	+	+	-	-14
S	M	20	2,413	28.5	-	+	-	1

<sup>a</sup> F, female; M, male.<sup>b</sup> 2<sup>nd</sup>, second generation.

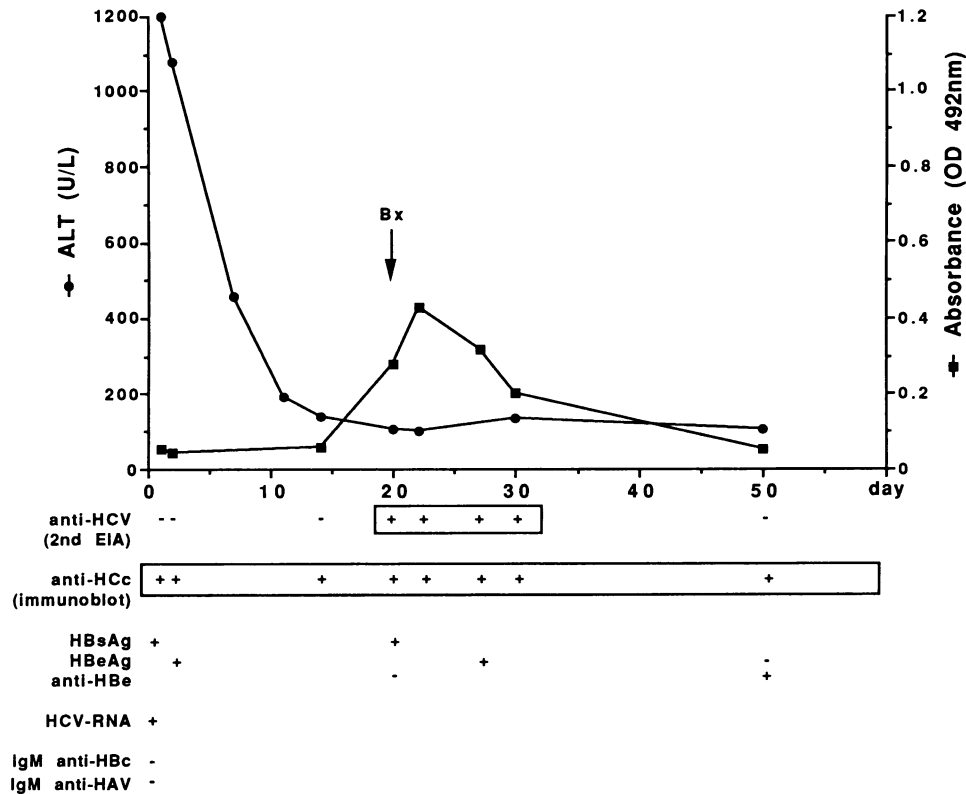


FIG. 2. Schematic illustration of data for a patient with HBV and HCV double infection. The x axis indicates days after admission. The arrow marks the day of the liver biopsy (Bx). The OD cutoff value for anti-HCc is 0.167. The S/N ratios (count ratios of tested specimens to negative cutoff values) for HBsAg on days 1 and 21 were 15,420/238 and 19,954/159, respectively; the S/N ratios for HBeAg on days 3, 26, and 50 were 1,530/821, 1,470/984, and 825/926, respectively; and the S/N ratios for anti-HBe on days 21 and 50 were 5,476/3,046 and 1,872/2,121, respectively.

intracanalicular cholestasis were noted. No HBsAg-positive cells nor HBeAg-positive cells could be stained immunologically. Interestingly, the absorbance obtained during the course of infection and eventually went below the cutoff value (0.167). However, the anti-HCc detected by Western blot was positive throughout the course of infection. The decreasing level of anti-HCV after the acute stage was surprising, and the reason for it was not known.

**DISCUSSION**

Serological diagnosis of acute hepatitis C has been hindered by two major problems. First, the interval between the peak ALT level and the appearance of anti-HCV antibody by EIA varies from one patient to another. In some patients, the antibody can appear as late as 4 to 6 months after the peak ALT level; in others, rapid appearance may happen within 10 weeks (10, 31). Second, diagnosis of newly acquired acute infection relies on seroconversion of anti-HCV from negative to positive, which means that serial serum samples from the same patient are required. When only the first serum sample from a patient with an elevated ALT level is available and it is positive for anti-HCV, one can never distinguish whether it is a newly acquired acute hepatitis C infection or a chronic hepatitis C infection with an acute exacerbation. This situation is slightly improved by the application of RT-PCR for HCV RNA. When the serum sample from a patient with an elevated ALT level is negative for anti-HCV but positive for HCV RNA

by RT-PCR, we may consider it a newly acquired acute hepatitis C infection. Unfortunately, this method is of limited clinical value. This procedure has to be performed in a very well-established laboratory, and interlaboratory variability is uncertain (10, 13). Transient or intermittent positive status for HCV RNA has also been observed for some newly acquired acute hepatitis C infections (1, 10, 12, 34). Thus, a series of serum samples is still required for accurate diagnosis. In a recent study, IgM anti-HCV has been proposed as a possible marker for acute hepatitis C. Unfortunately, this marker also appears in chronic patients with acute exacerbation (5). With these problems, one cannot make a prompt diagnosis of the early stage of an acute hepatitis C infection nor can one determine acute or chronic status from a single serum sample. Furthermore, clinical experiments in therapeutic measurement are greatly hindered by the lack of a tool for prompt and accurate diagnosis.

In this report, we have concentrated on subjects with acute hepatitis C. All of them had evidence of newly acquired infection. We engineered a recombinant HC<sub>c</sub> protein fragment to be the target antigen and performed immunoblots for detection of anti-HCc. In the healthy control group (group I), none of them was positive for anti-HCc by this test, which is consistent with the previous report that the carrier rate of anti-HCV antibody is around 1% in Taiwan (18). In the group with acute hepatitis A or B, only 4 of 22 samples (18%) were positive. The positive rate for anti-HCV antibody in subjects with elevated ALT levels is reported to be 10%, with C100-3 as the target antigen (18). The actual incidence should, therefore,

be higher than 10% when a more sensitive test is applied. In our case, subjects were diagnosed with acute hepatitis A or B infection. The positive rate for anti-HCV in this group has never been reported. One of these four subjects also had a positive result when a second-generation EIA was used. This patient was then confirmed either to be a concealed HCV carrier superimposed with an acute hepatitis B infection or to have an acute double viral infection. For the other three subjects (13%), it is less certain whether a low titer of anti-HC<sub>c</sub> was detected or a false-positive result was observed, though the former is more likely since there was no false-positive case among the healthy controls (group I). Among the acute HCV-infected subjects (group III), the positive rate for anti-HC<sub>c</sub> was quite high (12 of 19 [63%]), whereas the positive rate for anti-HCV by EIA was only 21% (4 of 19), which is consistent with the results of previous studies (23, 31, 32). Among the subjects with EIA-negative status, 53% (8 of 15) are still positive for anti-HC<sub>c</sub> by our Western blot assay. For these eight cases (patients F to L and S) (Table 3), serum samples were obtained within -19 to 18 days after the peak ALT level except for patient G (51 days). In fact, five of them were checked within 3 days of the peak ALT level. It is likely that a low titer of anti-HC<sub>c</sub> may appear in a quite early stage of acute hepatitis C. The titer is so low that it can be detected only by Western blot. Of the four subjects with EIA-positive status, all were also positive for anti-HC<sub>c</sub> by Western blot. Their serum samples were obtained -14, 0, 9, and 60 days after their peak ALT levels. The first three of these samples may represent those of patients with early seroconversion. The early appearance of anti-HC<sub>c</sub> in acute hepatitis C has also been reported by other researchers (10, 31), but Western blot has rarely been used in the studies (23, 32). In our case, only patients with evidence of seroconversion or with EIA-negative and RT-PCR-positive results were included. Thus, all of them were considered to have newly acquired infections. However, the positive results for anti-HC<sub>c</sub> by Western blot analysis with some of the EIA-negative serum samples (8 of 15) raised a remote possibility that the anti-HC<sub>c</sub>, though at a low titer, might have existed long before this episode of acute exacerbation and was not due to a new infection. Some of these subjects should then have been diagnosed as having chronic hepatitis C with acute exacerbation. To date, we cannot rule out this possibility. These data suggest that Western blot is more sensitive for detection of anti-HC<sub>c</sub> than the second-generation EIA. The sensitivity of RT-PCR seems to be even higher than that of Western blot (80 versus 63% positive) for early detection. However, four of our cases are included because of positive RT-PCR results. If these four subjects are excluded, the positive rate goes down to 73% (11 of 15). In our laboratory, the positive rate for HCV RNA in chronic HCV infection is over 90%. The lower positive rate for cases of acute hepatitis C is consistent with reports that intermittent and transient positive RT-PCR results may occur with acute hepatitis C (1, 10, 12, 34). Although RT-PCR is the most sensitive test, it cannot distinguish between the acute and chronic states of the disease.

The carrier rate of HBV in Taiwan is very high (29), which makes dual infection by HBV and HCV an important issue in this area. In this report, we also analyzed a series of serum samples from an HBV carrier superinfected with HCV. HB<sub>e</sub>Ag converted to negative after HCV infection, which is consistent with our previous observation (19). This phenomenon is possibly due to a suppression of replication of HBV by HCV but not to an exacerbation of HBV itself. The biopsy result was also compatible with the activity of HCV infection rather than HBV infection (26). Another interesting observa-

tion was that the absorbance by EIA for anti-HCV progressively decreased and eventually fell below the cutoff value after the onset of hepatitis attack. This is an interesting phenomenon which occurs only occasionally during the convalescent phase of acute hepatitis C but not during chronic hepatitis (23, 30). In this case, we do not yet know the significance of it, though convalescence from acute hepatitis C with continuing chronic hepatitis B may be one possible explanation. The anti-HC<sub>c</sub> detected by our immunoblot assay persisted throughout the course of infection, which may suggest that either a low titer of anti-HC<sub>c</sub> appears early in infection or the anti-HC<sub>c</sub> exists long before this episode. Again, we cannot judge whether the patient possesses two viruses (HBV and HCV) for a long time with an exacerbation of hepatitis C activity or is an HBV carrier with a newly acquired acute hepatitis C infection.

In summary, we have developed an immunoblot assay which can potentially be used as a complementary test to a second-generation EIA in the diagnosis of acute hepatitis C. Our study also suggests that a low titer of anti-HC<sub>c</sub> exists in an even earlier stage of acute hepatitis C infection; this titer cannot be detected by the second-generation EIA. A more sensitive test such as Western blot may be required for an early diagnosis of acute hepatitis C. In addition, there may be a significant number of patients who have chronic HCV infection and carry a low titer of anti-HC<sub>c</sub>. These cases may be diagnosed as newly acquired acute hepatitis C infections when these patients experience acute exacerbations. New serological tests should be developed in order to solve these problems. Patients with double infection by HBV and HCV are a very interesting subset. More cases need to be analyzed to obtain more information before we can explain the phenomenon observed during viral interaction.

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